MOLECULAR AND CELLULAR BIOLOGY, June 1996, p. 2977–2986 Vol. 16, No. 6
0270-7306/96/$04.00+0

Direct Repeats Bind the EcR/USP Receptor and Mediate Ecdysteroid Responses in *Drosophila melanogaster*

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Received 28 September 1995/Returned for modification 6 December 1995/Accepted 11 March 1996

The steroid hormone 20-hydroxyecdysone plays a key role in the induction and modulation of morphogenetic events throughout *Drosophila* development. Previous studies have shown that a heterodimeric nuclear receptor composed of the EcR and USP proteins mediates the action of the hormone at the transcriptional level through binding to palindromic ecdysteroid response elements (EcREs) such as those present in the promoter of the *hsp27* gene or the fat body-specific enhancer of the *Fbp1* gene. We show that in addition to palindromic EcREs, the EcR/USP heterodimer can bind in vitro with various affinities to direct repetitions of the motif AGGTCA separated by 1 to 5 nucleotides (DR1 to DR5), which are known to be target sites for vertebrate nuclear receptors. At variance with these receptors, EcR/USP was also found to bind to a DR0 direct repeat with no intervening nucleotide. In cell transfection assays, direct repeats DR0 to DR5 alone can render the minimum viral *tk* or *Drosophila Fbp1* promoter responsive to 20-hydroxyecdysone, as does the palindromic *hsp27* EcRE. In a transgenic assay, however, neither the palindromic *hsp27* element nor direct repeat DR3 alone can make the *Fbp1* minimal promoter responsive to premetamorphic ecdysteroid peaks. In contrast, DR0 and DR3 elements, when substituted for the natural palindromic EcRE in the context of the *Fbp1* enhancer, can drive a strong fat body-specific ecdysteroid response in transgenic animals. These results demonstrate that directly repeated EcR/USP binding sites are as effective as palindromic EcREs in vivo. They also provide evidence that additional flanking regulatory sequences are crucially required to potentiate the hormonal response mediated by both types of elements and specify its spatial and temporal pattern.

The steroid hormone 20-hydroxyecdysone (20E) coordinates morphogenetic events throughout the *Drosophila* life cycle (56). At the end of the third larval instar stage, 20E coordinates a complex genetic program leading to puparium formation and metamorphosis (for a review, see reference 2). As first evidenced by Ashburner et al. (7), a part of this program consists of a genetic cascade in which the ecdysteroid receptor directly induces the expression of a small number of early puff genes (11, 14, 28, 63), whose products in turn regulate the expression of a much larger set of late puff genes (19, 21, 27). Although early puff genes are expressed in primary response to ecdysteroids in all tissues during late third larval instar and puparium formation, extensive analysis revealed that they are transcribed in complex and differential waves (1, 25, 26). In addition to early puff genes, a number of primary ecdysteroid-induced genes with strict tissue-specific expression have also been isolated (discussed in reference 50; for a review, see reference 2).

How can a single hormonal signal direct temporally and spatially distinct genetic responses during late third larval instar and puparium formation? Diversity of ecdysteroid genetic responses may result from modulation of ecdysteroid receptor level or variability of its target site structure.

The active form of the ecdysteroid receptor is a heterodimer of two members of the nuclear receptor superfamily, the EcR and USP proteins (22, 32, 52, 64). Although 14 other *Drosophila* nuclear receptors have been identified (reviewed in reference 70; see also references 18, 70, and 79), only heterodimers of EcR and USP seem to be able to bind 20E and activate transcription through ecdysteroid response elements (EcREs) (69, 74, 75). While the *usp* gene is expressed essentially uniformly during the late third larval instar stage (23), transcription of the *EcR* gene varies, with peaks 10 h before and 6 h after puparium formation (1, 25). Moreover, the *EcR* gene encodes three EcR protein isoforms, A, B1, and B2, that are expressed differentially in larval and imaginal tissues (68). This suggests that at least part of tissue- and stage-specific ecdysteroid genetic responses could be dictated by modulations in the expression of the EcR proteins.

A very limited number of EcREs have been characterized as both in vitro binding sites for the EcR/USP heterodimer and in vivo functional hormone response elements (3 and references therein). These sites harbor a 13-bp palindromic sequence with a single intervening nucleotide. They present homology with inverted repeats of the motif PuG(G/T)TCA, known to be target sites for vertebrate nuclear receptors (for a review, see reference 46). However, palindromic EcR/USP binding sites admit large sequence variations and can be very degenerate (4, 5, 12, 40, 53). The recent discovery that the USP protein is both structurally and functionally related to the vertebrate 9-*cis* retinoic acid receptor (RXR) (69, 74), which promiscuously heterodimerizes with other nuclear receptors and activates transcription through direct repeats of the motif PuG(G/T)TCA separated by a variable number of nucleotides (DRn) (for reviews, see references 36 and 39), suggests that the repertoire of EcR/USP target sites is large enough to include sites composed of direct repeats.

Indeed, Horner et al. (24) have recently shown that the EcR/USP heterodimer can bind in vitro to DR3, DR4, and

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DR5. Examination of the Eip28/29 [Dist] EcRE reveals that its structure, which was first recognized as an imperfect palindrome (12), includes a degenerate direct repeat with 3-nucleotide spacing. Recent in vitro binding studies have demonstrated that both half sites of this DR3 are important for EcR/USP heterodimer binding to the Eip28/29 element (78). On the other hand, D'Avino et al. (13) have reported EcR/USP binding to a synthetic element that is referred to as a DR3 motif but whose structure actually corresponds to a DR4 motif according to standard nomenclature for the structure of DR elements (44, 73). In addition, those investigators have shown that this element is able to mediate 20E induction of a reporter construct transfected in a *Drosophila* cell line. They have also found that a direct repeat element with 11 intervening nucleotides occurring naturally in the coding sequence of the ecdysteroid-regulated *ng1* and *ng2* genes is also a functional EcRE by using in vitro binding and ex vivo transfection assays. These data suggest that in the context of a developing whole organism, the various directly repeated elements, in addition to palindromic elements, may function as EcREs and generate the diversity of the genetic responses to ecdysteroid. To address this question, we investigated the functionality of the directly repeated sites as EcREs and their capacity to mediate a hormonal response within the context of the *cis*-acting sequences of the *Fat body protein 1* (*Fbp1*) gene, a primary ecdysteroid response gene.

Transcription of the *Fbp1* gene takes place in the larval fat body exclusively and is triggered by the large ecdysteroid peak which occurs during the second half of the third larval instar stage (1, 41, 54). Deletion analyses have pointed to two important *cis*-regulatory sequences of *Fbp1*, a 70-bp enhancer (−69 to −138), which is sufficient by itself to specify the spatially and temporally correct ecdysteroid-controlled pattern of *Fbp1* expression (37), and a 32-bp sequence (−194 to −162) which amplifies at least fivefold the specific transcriptional response conferred by the enhancer (35). In vitro binding studies have shown that the *Fbp1* enhancer contains a pseudopalindromic EcR/USP binding site whose in vivo occupancy is dependent on a high concentration of ecdysteroids, as shown by genomic footprinting experiments (3).

Here we show that the EcR/USP heterodimer binds in vitro to direct repeats DR0 to DR5. In addition, we demonstrate the ability of these direct repeats to confer ecdysteroid responsiveness on minimal promoters in a cell transfection assay. We also show that the natural pseudopalindromic EcR/USP binding site in the *Fbp1* enhancer is required to mediate a fat body-specific ecdysteroid response in a transgenic assay and can be substituted by direct repeat DR3 or DR0 without changes in the spatial and temporal specificities of this hormonal response. This demonstrates that direct repeats, as well as palindromes, function in the context of the whole organism as efficient EcREs. The profound differences in structure between these two types of EcR/USP target sites do not seem, however, to dictate a spatial and temporal specificity of the transcriptional response they mediate.

MATERIALS AND METHODS

Oligonucleotides and plasmids. Double-stranded oligonucleotides used in gel retardation experiments and plasmid constructions are depicted in Fig. 1.

The Mt-EcR and Mt-USP expression vectors were a generous gift from M. Furia (13). The Mt-EcR expression vector contains the entire coding region of EcR-B1 cDNA pMK1 (68) subcloned into pRmHa-1 (10). The Mt-USP expression vector contains USP cDNA clone pZ7-1 (22) inserted into pRmHa-1.

For in vitro synthesis of the EcR protein, the *Bam*HI-*Hin*dIII fragment of the cDNA encoding the EcR-B1 isoform was subcloned in Bluescript KS+ to generate the EcR-Bluescript KS+ plasmid. The USP protein was synthesized from full-length cDNA clone pZ7-1 (a gift from Vincent Henrich).

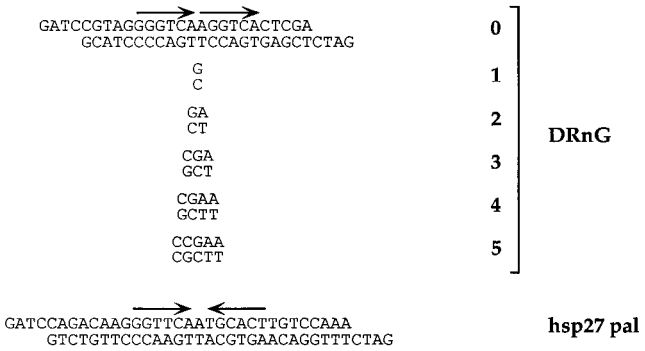

FIG. 1. Oligonucleotides used for DNA binding assays and plasmid constructs. The sequences of both strands of the oligonucleotides are shown. The last four nucleotides were left as 5′ overhangs for radioactive labeling of the DNA. The DRnG oligonucleotides contain direct repeat motifs identical to those used by Mader et al. (43) to study RAR, RXR, and TR DNA binding. The hsp27pal oligonucleotide contains the palindromic EcRE that lies upstream from the *D. melanogaster hsp27* promoter and confers 20-E inducibility upon it in transfection experiments (58).

Chloramphenicol acetyltransferase (CAT) reporter plasmids were constructed by inserting two copies of double-stranded oligonucleotide DR1G, DR2G, DR3G, DR4G, or DR5G (Fig. 1) in a head-to-tail orientation into the *Bam*HI site of ptkAT0 (15). The orientation and number of response elements were determined by suitable restriction analysis.

For construction of germ line transformation vector pBP1, the −68 to +80 fragment of the *Fbp1* promoter (47) was amplified by PCR with a 5′ primer flanked by *Bam*HI and *Not*I sites and a 3′ primer flanked by a *Kpn*I site. The amplified fragment was digested by *Bam*HI and *Kpn*I and inserted into the polylinker of germ line transformation vector pCasPerAUGβgal (71), resulting in an *Fbp1-lacZ* fusion gene with unique upstream *Bam*HI and *Not*I cloning sites (see Fig. 4A). For construction of pAEP1, the −194 to −69 region of the *Fbp1* promoter was PCR amplified with specific primers flanked by a *Not*I site, digested with *Not*I, and inserted into the unique *Not*I site of pBP1. Plasmids pAEδP1, pAED$_0$P1, and pAED$_3$P1 were derived from pAEP1 by site-directed mutagenesis of the *Fbp1* EcR/USP binding site between −92 and −106 in the *Fbp1* promoter (see Fig. 5). The structures of all of the constructs containing PCR amplified fragments or obtained by site-directed mutagenesis were confirmed by sequencing. Plasmids p2hspP1 and p10hspP1 were constructed by inserting 2 or 10 synthetic double-stranded hsp27pal oligonucleotides in a head-to-tail orientation into the *Bam*HI site of pBP1. Likewise, plasmids p2D$_3$P1 and p7D$_3$P1 were constructed by inserting two or seven double-stranded DR3G oligonucleotides in a head-to-tail orientation into the *Bam*HI site of pBP1 (see Fig. 4). The orientation and number of response elements were determined by suitable restriction analysis.

In vitro translation and protein extracts. After linearization of EcR-Bluescript KS+ by *Hin*dIII and pZ7-1 by *Cla*I, EcR-B1 and USP RNAs were transcribed (Stratagene) and translated in vitro in a rabbit reticulocyte lysate (Promega) in accordance with the manufacturer's instructions. Late-third-instar fat body nuclear extracts were prepared as described previously (3).

DNA binding assays. Gel retardation assays with in vitro-translated EcR and USP proteins were performed as previously described (23). Gel retardation assays with late-third-instar fat body nuclear extract were also performed as previously described (4). Supershift experiments were performed by adding 4 μl of EcR antibody AG10.2 (68) and 2 μl of USP antibody AG11 (29) to the reaction mixture.

Transient transfections. *Drosophila* Schneider 2 (S2) cells were cultured at 23°C in Schneider's medium (GIBCO) supplemented with 5% heat-inactivated fetal calf serum. Cells were DNA transfected with liposomes formed in the presence of *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP; Boehringer Mannheim) in accordance with the manufacturer's instructions. From 3×10^6 to 5×10^6 cells were incubated for 24 h in the presence of 20 μg of DNA, including 10 μg of a reporter plasmid and 5 μg each of the Met-EcR-B1 and Met-USP expression vectors and then cultured for an additional 24 h with or without 1 μM 20E. The EcR and USP expression vectors were used without addition of copper, because control experiments showed that the same transactivation was achieved without or with metal induction. Transfections with all constructs were repeated four times in different experiments.

Following treatment, the cells were collected by centrifugation and washed in phosphate-buffered saline and a cell extract was prepared as described by Gorman et al. (20). Protein concentrations were determined by the Bio-Rad protein assay. CAT and β-galactosidase activities in cell extracts were assayed as described by Simon and Lis (65) and Gorman et al. (20), respectively.

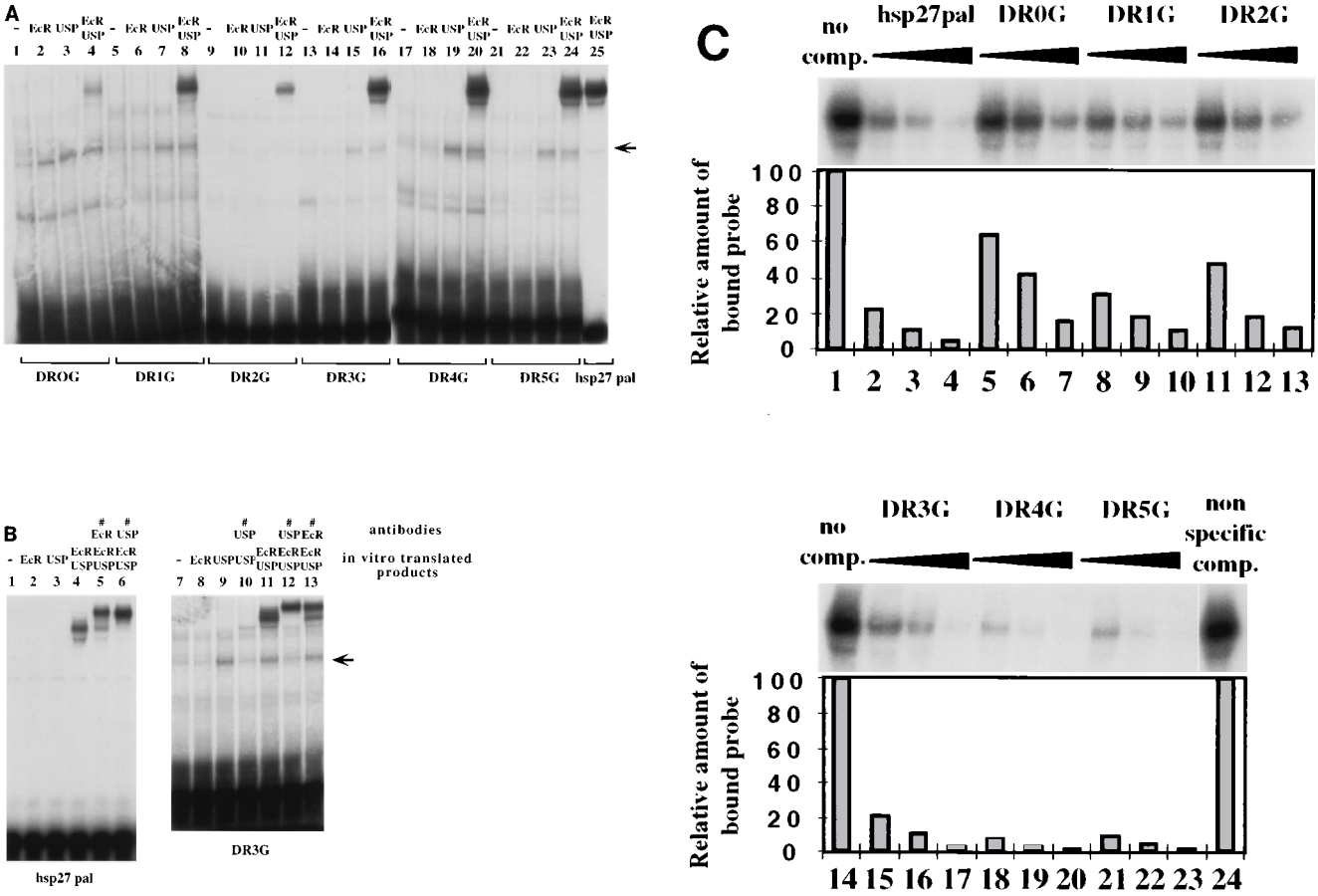

FIG. 2. Binding of the EcR and USP proteins to DRnG direct repeats. (A) DRnG and hsp27pal double-stranded oligonucleotides were used as radioactive probes in a gel shift assay in the presence of an unprogrammed rabbit reticulocyte lysate (−), the in vitro-translated EcR or USP protein, or a mixture of both, as indicated. The arrow indicates a band corresponding to the binding of the USP protein alone, which comigrates with a band due to nonspecific DNA binding activity in the rabbit reticulocyte lysate. (B) hsp27pal and DR3G radioactive probes were used as in panel A in a gel shift assay with the in vitro-translated EcR and USP proteins and in the presence (#) or absence of anti-EcR or anti-USP monoclonal antibodies. The arrow points to the band corresponding to the bound USP protein alone. (C) The EcR/USP-hsp27pal radioactive complex was formed in a gel shift assay (lane 1) and allowed to compete in the presence of increasing concentrations of unlabeled hsp27pal (lanes 2 to 4), DRnG elements (lanes 5 to 23), or the upper strand of the hsp27pal oligonucleotide as a nonspecific competitor (lane 24). The specific competitors were used at 5-, 10-, and 20-fold molar excesses, respectively. The nonspecific competitor was used at a 20-fold molar excess. comp., competitor.

Germ line transformations and histochemical staining. DNAs of the germ line transformation vector (250 μg/ml) and helper plasmid Δ2-3 (50 μg/ml) were microinjected into embryos of a w^{1118} recipient stock (61). w^{+} transformants were screened for eye color. The number of transgenes in each line was determined by Southern blotting.

Larvae were dissected in phosphate-buffered saline (pH 7.5). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining of dissected tissues for β-galactosidase activity was performed as previously described (6).

RESULTS

The EcR/USP heterodimer binds with various affinities to directly repeated sites. The EcR and USP proteins were produced in vitro by using a rabbit reticulocyte lysate translation system, and their binding to a series of elements containing direct repetitions of the PuGGTCA motif (hereafter termed DRnG, where n indicates the number of spacer nucleotides separating the repeated motifs [Fig. 1]) was investigated by using a gel retardation assay. As a control, binding of the EcR and USP proteins was also tested by using the canonical palindromic EcRE from the *hsp27* gene (hereafter referred to as hsp27pal) as a probe (Fig. 1). When mixed together, the EcR and USP proteins formed a predominant, slowly migrating complex with direct repeats DR0G to DR5G and the hsp27pal elements (Fig. 2A, lanes 4, 8, 12, 16, 20, 24, and 25). This complex corresponds to the binding of an EcR/USP heterodimer to DNA, as seen by supershifts with anti-EcR and anti-USP monoclonal antibodies (Fig. 2B and data not shown).

When the EcR protein was tested alone, no binding to the DRnG direct repeats or to hsp27pal was detected above the nonspecific DNA binding background due to rabbit reticulocyte lysate components (compare lanes 2, 6, 10, 14, 18, and 22 with control lanes 1, 5, 9, 13, 17, and 21 in Fig. 2A and lanes 1 and 2 in Fig. 2B). By contrast, when tested alone with DR0G, DR1G, DR3G, DR4G, and DR5G, the USP protein gave rise to the formation of a weak retarded complex with fast electrophoretic mobility that comigrated with a nonspecific complex due to a rabbit reticulocyte lysate component (Fig. 2A, lanes 3, 7, 15, 19, and 23). This weak complex was supershifted by an anti-USP monoclonal antibody (Fig. 2B, lanes 9 and 10 and data not shown), indicating that it corresponds to the USP protein bound to direct repeats. No binding of the USP protein alone was detected with either the DR2G or the hsp27pal probe.

The EcR/USP DNA binding affinity for DRnG elements was further analyzed in competition experiments (Fig. 2C). EcR/USP binding was carried out in the presence of the radiolabeled hsp27pal element, and the EcR/USP-DNA complex that

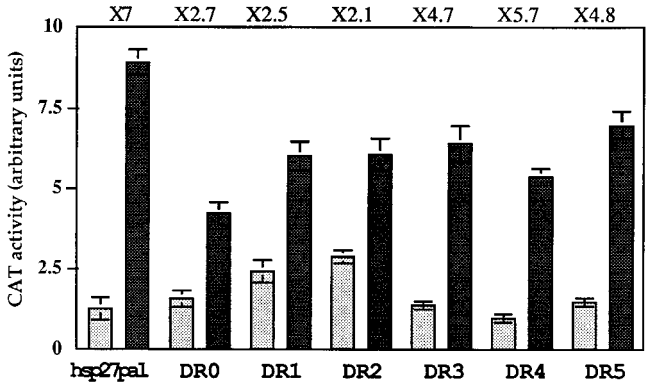

FIG. 3. Comparison of 20E transcriptional induction conferred by the hsp27pal and DRnG elements on a *tk*-CAT reporter construct. Two copies of the hsp27pal or DRnG element (Fig. 1) were inserted in tandem upstream of the herpes simplex virus TK promoter in the ptkAT0 reporter plasmid (see Materials and Methods). The resulting reporter plasmids were cotransfected in S2 cells together with the MT-EcR and Mt-USP expression vectors, and 10^{-6} M 20E was added (▨) 24 h before harvesting of the cells and measurement of CAT activity. No 20E induction was observed upon transfection of a control plasmid carrying only a *tk*-CAT reporter gene without any elements inserted upstream (data not shown). Standard errors (n = 4) and 20E induction ratios are indicated. ▨, no 20E.

formed was eliminated by the presence of increasing concentrations of an unlabeled hsp27pal or DRnG element. Efficiencies of competition indicate that the DNA binding affinity of EcR/USP for DRnG and hsp27pal elements follows the order DR4G > DR5G > DR3G = hsp27pal > DR1G > DR2G > DR0G.

We also tested the binding of EcR/USP to direct repeats of the motif PuGTTCA, referred to as DRnT, which differs from DRnG by only a T instead of a G at the third position. When tested alone, neither the EcR nor the USP protein bound to any of the DRnT elements. In contrast, the EcR/USP heterodimer bound with various affinities to DR1T, DR2T, DR3T, DR4T, and DR5T. No EcR/USP binding to DR0T was detected (2a).

Direct repeats confer 20E responsiveness on minimal promoters in a *Drosophila* cell line. As DRnG elements are in vitro EcR/USP binding sites, we investigated whether they can confer 20E inducibility on a reporter gene in cultured cells. For this purpose, we made a series of constructs carrying two copies of the DRnG elements inserted upstream from the minimal thymidine kinase promoter fused to the CAT reporter gene. As a reference, two copies of the hsp27pal element were inserted upstream of the same reporter gene. In preliminary transfections of *Drosophila* S2 cells with the latter construct, threefold 20E induction was observed (data not shown). Upon cotransfection with EcR and USP expression vectors, the 20E inducibility of this construct reached a more workable sevenfold level (Fig. 3). Hence, all constructs carrying two copies of the DRnG elements were tested upon cotransfection with the EcR and USP expression vectors.

Four- to fivefold stimulation of transcriptional activity by 20E treatment was observed for reporter constructs carrying two copies of the DR3G, DR4G, and DR5G elements. Likewise, the transcriptional activities of the DR0G, DR1G, and DR2G reporter genes were enhanced in the presence of hormone, but to a lesser extent of about two- to threefold. These results demonstrate that direct repeats DR0G to DR5G act as EcREs in *Drosophila* cells. The level of stimulation of their transcriptional activity is in good correlation with their in vitro EcR/USP binding affinity. However, although the hsp27pal element is bound in vitro by the EcR/USP heterodimer with a lower affinity than DR4G and DR5G elements, it confers better stimulation by 20E than any of the DRnGs (Fig. 3). This result suggests that not only the EcR/USP relative binding affinity but also the structure of the EcR/USP binding site is important for the ecdysteroid response.

To determine whether a direct repeat element can confer ecdysteroid responsiveness on a *Drosophila* promoter, we focused on the DR3G element because its binding affinity appears to be equivalent to that of the hsp27pal element. Starting with the parental reporter construct pBP1, two or seven copies of the DR3G element were placed upstream of the *Fbp1* −68 to +80 minimal promoter fused to the *lacZ* reporter gene to generate the p2D_3P1 and p7D_3P1constructs, respectively (Fig. 4A). In addition, 2 or 10 copies of the hsp27pal element were fused in front of the *Fbp1* (−68 to +80)-*lacZ* reporter gene to generate the control p2hspP1 and p10hspP1 constructs (Fig. 4A). S2 cells were cotransfected with EcR and USP expression vectors and the pBP1, p2D_3P1, p7D_3P1, p2hspP1, or p10hspP1 construct. β-Galactosidase activity was not stimulated by 20E

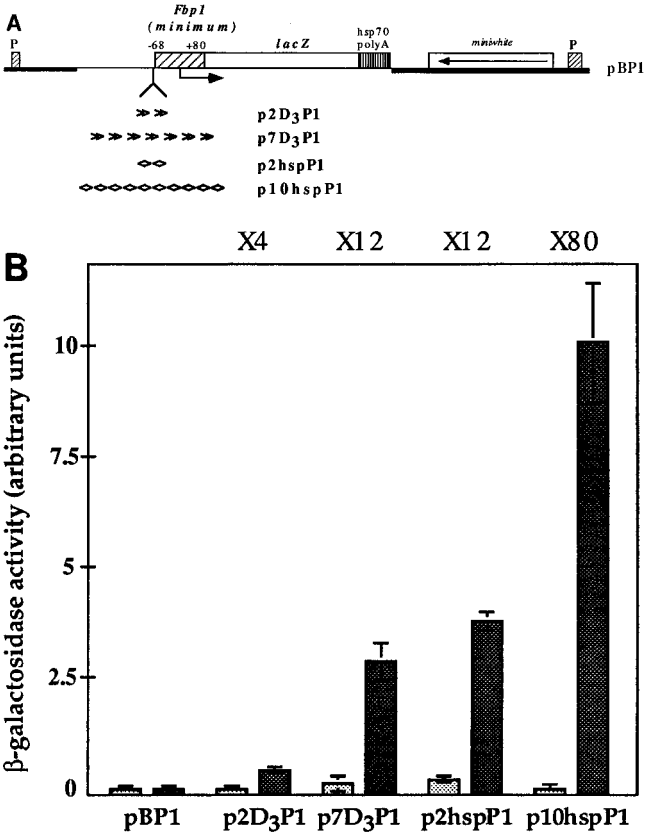

FIG. 4. Comparison of 20E transcriptional induction conferred by the hsp27pal and DR3G elements on an *Fbp1-lacZ* reporter construct. (A) Schematic structure of the pBP1 construct and its derivatives. The pBP1 vector was constructed by inserting the *Fbp1* minimum promoter (−68 to +80) upstream from the *lacZ* gene into the pCasPerAUGβgal vector (71). p2D_3P1 and p7D_3P1 were derived from pBP1 by inserting, upstream from the *Fbp1* promoter, 2 and 7 copies of the DR3G element or 2 and 10 copies of the hsp27pal element, respectively. All of these reporter constructs contain a *miniwhite* selection gene and P transposon terminal sequences, making them ready to use for germ line transformation in addition to a cell transfection assay. (B) The *Fbp1-lacZ* reporter plasmids were cotransfected in S2 cells together with the Mt-EcR and Mt-USP expression vectors as described in the legend to Fig. 3. Specific β-galactosidase activity is shown in arbitrary units. Standard errors (n = 4) and 20E induction ratios are indicated. ▨, plus 20E; ▨, minus 20E.

treatment in transfections with the parental pBP1 construct (Fig. 4B). In contrast, 4- and 12-fold stimulation of β-galactosidase activity by 20E treatment was observed for constructs p2D$_3$P1 and p7D$_3$P1, respectively. This result shows that a DR3G element confers ecdysteroid inducibility on a *Drosophila* promoter in cultured cells which is proportional to the number of copies of DR3G. However, 12- and 80-fold stimulation of β-galactosidase activity by 20E treatment was observed with p2hspP1 and p10hspP1, respectively (Fig. 4B), showing that the hsp27pal element behaves as a stronger EcRE than the DR3G element under these conditions.

DR3G and hsp27pal elements are not sufficient to confer ecdysteroid response on the Fbp1 promoter during larval and pupal stages. Following the demonstration that direct repeats DR0G to DR5G act as EcREs in the *Drosophila* S2 cell line, the question arose as to whether they can also confer ecdysteroid inducibility on a minimal promoter by themselves in the context of a whole developing organism.

After injection of the pBP1, p7D$_3$P1, and p10hspP1 constructs (Fig. 4A) into preblastoderm embryos, several transgenic lines were established for the *Fbp1-lacZ*, 7DR3-*Fbp1-lacZ*, and 10hsp27-*Fbp1-lacZ* constructs. Expression of the *lacZ* reporter gene was analyzed by X-Gal staining in at least three independent lines. In the control *Fbp1-lacZ* transgenic lines, as well as in the 10hsp27-*Fbp1-lacZ* transgenic lines, no significant β-galactosidase activity was detected in embryos; first-, second-, or third-instar larvae; or pupae (see Fig. 6C; data not shown). Likewise, no β-galactosidase activity was detected during larval and pupal development of the 7DR3-*Fbp1-lacZ* transgenic lines (see Fig. 6D; data not shown). In contrast, transient and strong expression of the 7DR3-*Fbp1-lacZ* transgene was observed in the more dorsal cells of the amnioserosa during stages 12 and 13 of embryogenesis (see Fig. 6H). Hence, the DR3G and hsp27pal elements appear to be insufficient in the animal to place the *Fbp1* minimal promoter under the control of the ecdysteroid pulses which occur during larval and pupal development. On the other hand, expression of the 7DR3-*Fbp1-lacZ* transgene during embryogenesis could correspond to a specific response to the ecdysteroid pulse that occurs in embryos at the time of germ band shortening (45, 57).

Direct repeats DR0 and DR3 can substitute for a palindromic EcRE for determining ecdysteroid responsiveness of the Fbp1 gene enhancer in third-instar larvae. The inability of the DR3G and hsp27pal elements to drive transcriptional activity of the *Fbp1* minimal promoter in animals strongly contrasts with their ability to confer 20E responsiveness on the same promoter in cultured *Drosophila* cells. We reasoned that this may reflect the requirement of *cis*-acting flanking sequences in addition to EcR/USP binding sites to make up a functional EcRE in larvae. To test this hypothesis, we analyzed the in vivo ecdysteroid response activity of EcR/USP binding sites in the context of the *cis*-regulatory sequences of the *Fbp1* ecdysteroid-responsive gene (Fig. 5).

We first mutagenized the 11-bp pseudopalindromic EcR/USP target site between positions −92 and −102 in the pAEP1 construct by introducing into this site a mutation that had been shown previously to abolish EcR/USP binding in vitro (3). No β-galactosidase activity was detected by X-Gal staining of the fat body from third-instar larvae transgenic for the resulting pAEδP1 construct (Fig. 6B). Similarly, no β-galactosidase activity was detected in other tissues at this stage or at any other developmental stages. This result demonstrates that the natural EcR/USP binding site localized in the *Fbp1* enhancer is essential for its activity in vivo.

We then tested the effect of replacing this EcR/USP binding site by direct repetition DR0G or DR3G. Plasmids pAED$_0$P1

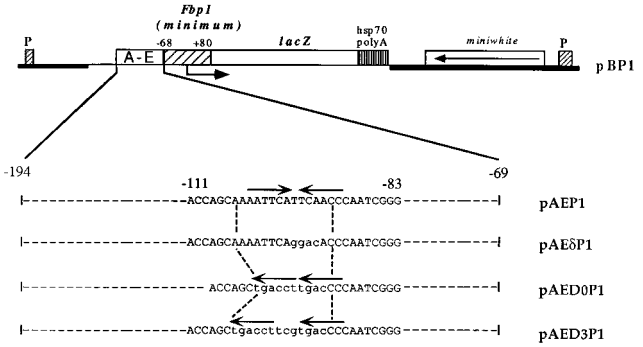

FIG. 5. Constructs used for transgenesis of *lacZ* reporter genes under the control of wild-type or mutated *Fbp1* enhancers. Sequences of the wild-type pseudopalindromic (pAEP1), mutated δ (pAEδP1), or directly repeated (pAED0P1 and pAED3P1) EcR/USP binding sites in the −194 to −69 amplifier-enhancer *Fbp1* region (A to E) used in transgenic assays are depicted. The mutated nucleotides are indicated by lowercase letters. The δ mutation has been shown to abolish in vitro EcR/USP binding to the *Fbp1* enhancer (3).

and pAED$_3$P1 were constructed by replacing the pseudopalindromic *Fbp1* EcR/USP binding site at −92 with direct repetition DR0G or DR3G (Fig. 5), and transgenic lines for the resulting AED$_0$P1-*lacZ* and AED$_3$P1-*lacZ* constructs were established. Both series of transgenic lines specifically exhibited strong β-galactosidase activity in the fat body of late-third-instar larvae that was indistinguishable from the β-galactosidase activity exhibited by transgenic lines for the wild-type AEP1-*lacZ* construct (Fig. 6A, E, and F). No expression of the AED$_0$P1-*lacZ* reporter transgene was, however, detected in other tissues at this stage or at any other developmental stage. In contrast, in amnioserosa cells, transient expression of the AED$_3$P1-*lacZ* transgene which was very similar to that exhibited by the 7DR3-*Fbp1-lacZ* transgene was observed (Fig. 6G and H). These results show that direct repeats DR0G and DR3G can replace the pseudopalindromic natural EcR/USP binding site in the *Fbp1* enhancer and mediate in this context a fat body-specific response to ecdysteroids in third-instar larvae. In addition, they strongly suggest that DR3G dictates specific expression in amnioserosa cells during embryogenesis.

Fat body nuclear factors binding to direct repeats DR0G to DR5G. The demonstration that DR0G and DR3G are able to mediate an ecdysteroid response in late-third-instar fat bodies prompted us to test in a gel shift assay whether the direct repeats are actual target sites for the EcR/USP receptor in this tissue.

As reported previously, the palindromic hsp27pal element, when used as a radioactive probe in the presence of late-third-instar fat body nuclear extract, gives rise to a sharp retarded band corresponding to the binding of the fat body EcR/USP heterodimer (Fig. 7A, lane 19). The complete supershift of this band in the presence of either an anti-EcR or an anti-USP antibody indicates that only the EcR and USP proteins participate in the formation of this complex (Fig. 7A, lanes 20 and 21). Direct repeats DR0G to DR5G also gave rise to retarded complexes with about the same electrophoretic mobility as that formed with hsp27pal (Fig. 7A, lanes 1 to 18). Although in all cases these complexes appeared much more diffuse, complete self-competitions in the presence of unlabeled probes demonstrated that they are sequence specific (Fig. 7B). However, they were only partially supershifted in the presence of anti-EcR and anti-USP antibodies. The proportion of anti-EcR or anti-USP supershifted complexes varied with the probe in the order DR5G > DR3G > DR4G > DR1G > DR2G > DR0G. Only

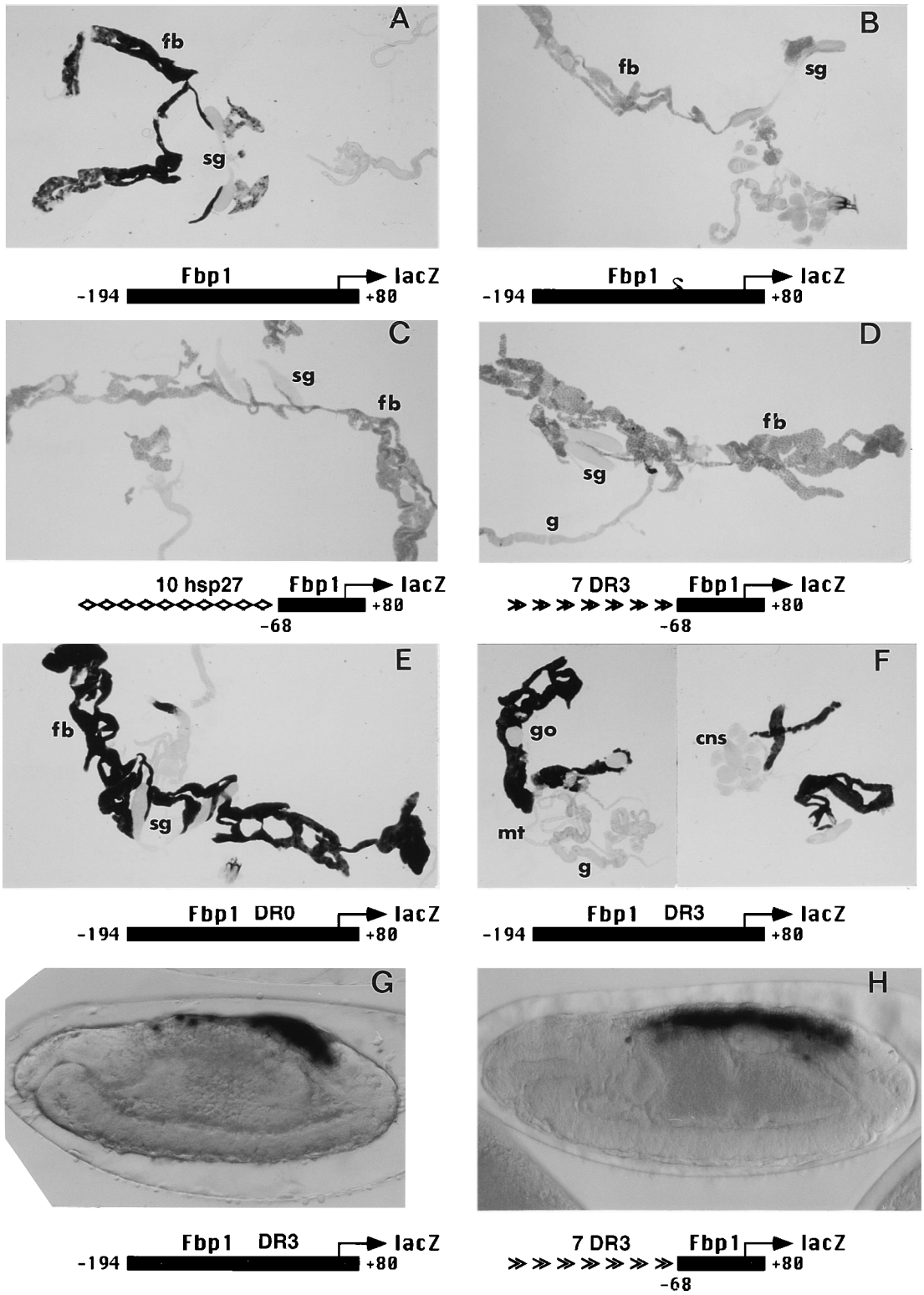

FIG. 6. Analysis of the expression conferred in vivo by palindromic or directly repeated EcR/USP binding sites by using *Fbp1-lacZ* transgenes. The simplified structures of the constructs used to establish transgenic lines are indicated below the panels: A, pAEP1; B, pAEδP1; C, p10hspP1; D and H, p7D_3P1; E, pAED_0P1; F and G, pAED_3P1. See Fig. 5 for the detailed sequences of the palindromic or directly repeated EcR/USP binding sites in the constructs. Dissected tissues (fat body [fb], salivary glands [sg], gut [g], gonads [go], central nervous system [cns], and Malpighian tubules [mt]) from late-third-instar larvae (A to F) or stage 13 embryos (G and H) were histochemically stained for β-galactosidase activity.

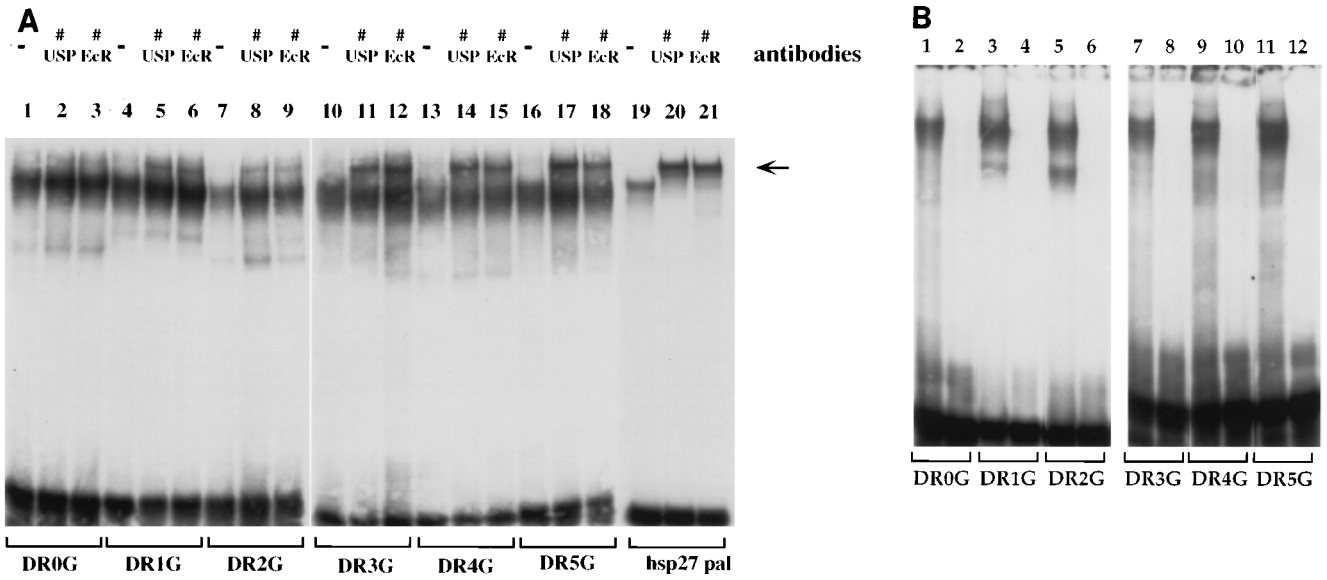

FIG. 7. Analysis of DRnG binding activities in late-third-instar fat body nuclear extracts. (A) hsp27pal and DRnG elements were used as radioactive probes in a gel shift assay with a nuclear extract from a late-third-instar fat body in the presence of anti-EcR or anti-USP monoclonal antibodies. The arrow indicates the supershifted complexes. (B) Self-competition of DRnG element binding activities. DRnG radioactive probes were used as for panel A in a gel shift assay with a nuclear extract from a late-third-instar fat body in the absence (lanes 1, 3, 5, 7, 9, and 11) or presence (lanes 2, 4, 6, 8, 10, and 12) of a 200-fold molar excess of unlabeled probe.

a very small proportion of the complex formed in the presence of DR0G was supershifted by anti-EcR or anti-USP antibodies.

Taken together, these results show that direct repeats DR0G to DR5G are bound by the EcR and USP proteins in the fat body nuclear extract with the same order of binding affinity as when bound by in vitro-translated EcR and USP proteins. However, contrary to the hsp27pal element, they appear to be bound by other, unidentified factors, raising the possibility that direct repeats are target sites for other combinations of nuclear receptors present in the larval fat body.

DISCUSSION

The EcR/USP heterodimer has a specific binding element repertoire. The functional characterization of EcR/USP target sites occurring naturally in the *cis*-regulatory sequences of ecdysteroid-responsive genes (3–5, 12, 15, 40, 42) led to the definition of EcREs as imperfect palindromic structures. However, recent studies have shown that EcR/USP binds in vitro to DR3G, DR4G, and DR5G elements (24) and that directly repeated motifs separated by 4 or 11 intervening nucleotides can mediate ecdysone response in a cell transfection assay (13). Here, we extend these results and show that the EcR/USP heterodimer can efficiently bind to direct repetitions of the AGGTCA motif separated by 0 to 5 nucleotides.

We found that the order of preference of EcR/USP for DRnG is DR4G > DR5G > DR3G > DR1G > DR2G > DR0G. Binding to directly repeated elements has been extensively established for other nuclear receptors such as heterodimers between RXR, the vertebrate receptor most related to USP, and TR, VDR, and RAR. For example, RXR/TR binds preferentially to DR4G but also to DR3G and DR5G and the RXR/RAR heterodimer binds to DR1G through DR5G, with the order of preference DR5G = DR4G > DR2G > DR1G > DR3G (43). Hence, by analogy with the situation encountered with vertebrate nuclear receptors, it is not surprising to find that the EcR/USP heterodimer can bind to different direct repeats. However, the order of preference of EcR/USP for the DRnGs is clearly different from that of the RXR, RAR, TR, and VDR receptors (17, 30, 43, 44, 66).

We have shown that EcR/USP can bind to DR0G. The ability of EcR/USP to bind to such closely spaced motifs is unique among the members of the nuclear receptor superfamily. In fact, steric hindrance at the dimerization interfaces in the DNA binding domains seems to exclude the binding of RXR/RXR to DR0, of RXR/RAR to DR0 and DR1, and of RXR/TR to DR0, DR1, and DR2 (43, 55, 77). In contrast to these receptors, EcR and USP must have flexible dimerization interfaces that allow their binding to DR0.

Binding of the USP protein alone to a direct repeat element with 11 intervening nucleotides has been previously reported by D'Avino et al. (13). In this study, we found that USP alone is also able to bind, with variable affinity, to DR0G, DR1G, DR2G, DR3G, DR4G, and DR5G. Our data do not allow us to discriminate whether this binding corresponds to a USP monomer or a USP/USP homodimer. However, the fact that the binding efficiency of the USP protein alone varies with the spacing between the repeated motifs suggests that USP binds to DNA as a homodimer. As previously reported, this binding does not occur with pseudopalindromic elements such as hsp27pal (Fig. 2B) (69, 75), the Fbp1-D EcRE (3), or the Lsp2 EcRE (5). Likewise, no binding of the USP protein alone to DRnT was observed (2a). Hence, these data suggest that binding of USP monomers or homodimers could occur with a particular subset of regulatory elements and mediate a specific hormonal response. However, binding of USP alone to DRnG is at least 1 order of magnitude less efficient than binding of the EcR/USP heterodimer, and its biological significance remains to be demonstrated.

Direct repeats mediate differential ecdysteroid response in cultured cells. Our cell transfection experiments demonstrate that direct repeats DR1G to DR5G can confer ecdysteroid responsiveness on a reporter gene. This result extends the finding by D'Avino et al. that a synthetic element including a DR4G motif can mediate ecdysone induction in a *Drosophila* Kc cell line. Upon cotransfection with EcR and USP producer

plasmids, maximal four- to fivefold 20E stimulation of the minimum *tk* promoter was observed with direct repeats DR4G, DR5G, and DR3G. These elements are those with the highest EcR/USP binding affinity, suggesting that the strength of the interaction between the EcR/USP heterodimer and DNA determines, in part, the intensity of the ecdysteroid genetic response. However, it must be stressed that palindromic element hsp27pal confers 20E stimulation that is higher than the stimulation conferred by any of the DRnG direct repeats. This indicates that not only EcR/USP binding affinity but also the structure (palindromic or directly repeated) of the element determines its ability to mediate an ecdysteroid response in vivo. Recent studies have shown that distinct dimerization interfaces are recruited when heterodimers of nuclear receptors are bound to palindromes or differently spaced direct repeats (76, 77), resulting, in some cases, in differential hormonal ligand sensitivity or differential transactivation (16, 34, 62). Our results support the view that the structure of the hormone response element determines the structure of the DNA-bound nuclear receptor and plays a role in its transactivation function.

We also tested the ability of DR3G to confer ecdysteroid responsiveness on the minimum *Drosophila Fbp1* promoter. Upon cotransfection with EcR and USP producer plasmids, 20E elicited fourfold stimulation of the 2DR3G-*Fbp1-lacZ* reporter gene, a stimulation level comparable to that found with the 2DR3G-*tk*-CAT construct. The 20E response of 7DR3G-*Fbp1-lacZ* was further enhanced, producing 12-fold stimulation. These data show that 20E stimulation of the *lacZ* reporter gene is proportional to the number of DR3G elements upstream of the *Fbp1* promoter, further establishing the role of DR3G as an EcRE. Interestingly, 20E elicited 12- and 80-fold stimulation of the 2hsp27-*Fbp1-lacZ* and 10hsp27-*Fbp1-lacZ* constructs, respectively. Hence, the difference in strength between the DR3G and hsp27pal EcREs is more marked in the context of the minimum *Fbp1* promoter than in the context of the *tk* promoter (compare Fig. 3 and 4B), suggesting a role of promoter context in modulating the 20E response conferred by EcREs.

Palindromic or directly repeated EcR/USP binding sites are not sufficient to mediate ecdysteroid responses in animals during larval and pupal development. *Drosophila* germ line transformation offers a unique opportunity to study the hormonal response of a reporter gene in the context of a highly integrated and developing organism. We found that DR3G direct repeats, either alone or in the context of the *Fbp1* enhancer, can specifically drive the expression of *lacZ* reporter transgenes in amnioserosa cells at stages 12 and 13 of embryogenesis. Interestingly, this period is marked by an ecdysteroid pulse (45), suggesting that DR3G-mediated *lacZ* expression in embryos could correspond to a specific ecdysteroid response. In contrast, the minimal *Fbp1* promoter under the control of either palindromic hsp27pal or DR3G elements is not expressed during larval and pupal *Drosophila* development. This absence of expression is particularly striking because the 10hsp27-*Fbp1-lacZ* and 7DR3-*Fbp1-lacZ* reporter genes do respond to 20E in cultured cells and dramatic ecdysteroid pulses occur during the third larval and prepupal stages (33, 57) which trigger expression of a large number of ecdysteroid-responsive genes (2). Moreover, all of the in vitro-characterized ecdysteroid receptor binding sites have been isolated from regulatory sequences of genes that respond to 20E during the third larval instar stage (3, 5, 12, 13, 42, 58). Our data therefore strongly suggest that in developing flies, genetic responses to ecdysteroids require other *cis*-regulatory sequences in addition to either palindromic or directly repeated EcR/USP binding sites.

Our results contrast with those obtained with similar transgenic assays for hormone response element activity in the mouse system. Several studies have shown that the directly repeated *trans*-retinoic acid response element present in the RARβ gene, when placed either upstream of an *hsp68* or a *tk* minimum promoter, can direct specific spatial and temporal expression of a *lacZ* transgene in mouse embryos and adults, without the need for additional *cis*-regulatory sequences (9, 59, 72). This may reflect unexpected intrinsic differences in hormonal regulation between vertebrates and invertebrates. However, it is interesting that patterns of expression of both of these *lacZ* transgenes are not strictly identical and only partially overlap with the pattern of expression of the endogenous RARβ gene (48, 49, 60). These results underline the fact that any choice of natural promoter might bias the pattern of reporter gene expression in a transgenic assay, since the cointeraction of activated nuclear receptors with other transcription factors that might bind to a basal promoter is not very well understood. They also show that in vertebrates, hormonal responses at particular developmental stages or in particular tissues may require other *cis*-regulatory sequences in addition to hormone response elements.

Direct repeats may function in transgenic animals as efficient EcREs when flanked by additional *cis*-regulatory sequences. By using fat body nuclear extracts for in vitro binding studies, we have demonstrated that the *Fbp1* enhancer is a binding target for the EcR/USP heterodimer and at least six other fat body nuclear factors (3). Thus, the *Fbp1* enhancer appears as a complex and composite regulatory element. In this report, we show that a mutation of the pseudopalindromic EcR/USP binding site in the *Fbp1* enhancer completely abolishes its ability to confer a fat body-specific ecdysteroid response on a *lacZ* reporter transgene. This result is evidence that in vivo binding of the ecdysteroid receptor to the *Fbp1* enhancer is essential to its EcRE function. However, replacement of the natural pseudopalindromic EcR/USP binding site with either a DR0G or a DR3G element does not alter this function, providing further evidence that the binding of EcR/USP has a key role in the ability of the *Fbp1* enhancer to mediate the ecdysteroid response. Most importantly, it shows that direct repeats do function in vivo as efficient EcREs, at least when placed in the *Fbp1* enhancer environment. In our gel shift analysis with fat body nuclear extracts, direct repeats were bound not only by EcR and USP proteins but also by other, unidentified factors. This leaves open the possibility that the ecdysteroid response mediated by direct repeats DR0G and DR3G in the fat body involves *trans*-regulatory factors distinct from the EcR/USP heterodimer. Apart from EcR and USP, five other *Drosophila* nuclear receptors, E75, E78, βFTZ-F1, DHR3, and DHR39 (8, 31, 38, 51, 63, 67), are known to be involved in the 20E genetic cascade activated at the onset of metamorphosis. Recently, Horner et al. (24) tested the binding of these seven nuclear receptors, either alone or in pairwise combinations, to DR3G, DR4G, and DR5G. However, they found that only EcR/USP heterodimers bind to these elements as heterodimers. Hence, if regulatory factors other than the EcR/USP heterodimer are involved positively in the ecdysteroid response mediated by the DR3G element, they remain to be identified. On the other hand, it has been recently reported that the *Drosophila* nuclear receptor Svp (78) and the newly identified orphan receptor XR78E/F-DHR78 (18, 79) may inhibit ecdysone signaling pathways. These receptors bind preferentially to a DR1 element but are also able to bind to a DR3 element. It would therefore be interesting to investigate whether they can interfere with the DR3-mediated ecdysteroid response, as revealed by our *lacZ* transgenes.

It is particularly striking to find that in the *Fbp1* enhancer environment in animals, palindromic or directly repeated sites with different structures and EcR/USP affinities mediate ecdysteroid responses with strictly conserved tissue and stage specificity. In particular, the DR0G element has much lower EcR/USP binding affinity than the DR3G element and is responsible for lower 20E induction of the *tk*-CAT reporter gene in cell transfections. However, both of these directly repeated elements can replace the natural pseudopalindromic EcR/USP binding site of the *Fbp1* enhancer and drive a fat body-specific ecdysteroid response similar to that conferred by the wild-type *Fbp1* enhancer. These findings emphasize the general importance of testing activities of hormone response elements not only in cultured cell systems but also in vivo in transgenic animals and have two important implications. First, they indicate that *Drosophila* ecdysteroid response tissue and stage specificities do not depend simply on the structure or affinity of EcR/USP target sites. Second, they underline the key role of the regulatory sequences flanking the EcR/USP binding site in the *Fbp1* enhancer. Our recent observation (4a) that a mutation of the α site (3) flanking the EcR/USP binding site and involved in the binding of three fat body nuclear factors completely abolishes the ability of the *Fbp1* enhancer to mediate the ecdysteroid response in transgenic lines adds strong support to this idea. Similarly, Lehmann and Korge (40) have shown that 20E induction of the *sgs-4* gene in salivary glands requires binding of the SEBP-3 factor to a site adjacent to an EcR/USP binding site. Taken together, these results suggest that synergy between an ecdysteroid receptor, bound to either a palindromic or a directly repeated site, and other transcription factors bound to additional *cis*-regulatory sequences is required to mediate tissue-specific ecdysteroid responses in the context of a developing organism.

ACKNOWLEDGMENTS

We are grateful to M. Arbeitman and D. S. Hogness for the gift of the anti-EcR AG10.2 antibody, to M. J. Shea and F. C. Kafatos for the gift of the anti-USP AB11 antibody, and to P. P. D'Avino and M. Furia for the gift of Mt-EcR and Mt-USP expression vectors. We gratefully acknowledge C. Elke, M. Lezzi, P. P. D'Avino, M. Furia, and C. S. Thummel for sharing information during the course of this work. We thank L. Dinan for helpful discussions and critical reading of the manuscript.

This work was supported by grants to J.-A. Lepesant from the Centre National de la Recherche Scientifique, the Association pour la Recherche sur le Cancer (6294), and the Ligue Nationale contre le Cancer.

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